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(12) (19) (CA) **Demande-Application**

(21) (A1) **2,286,694**
(86) 1998/04/09
(87) 1998/10/22

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(51) Int.Cl.[6] C12N 15/55, C12P 7/42, C12N 9/18, D21H 17/00, A23K 1/165, C12P 19/00
(30) 1997/04/14 (9707540.2) GB

(54) **ESTERASE D'ACIDE PHENOLIQUE ET UTILISATION DE CETTE DERNIERE**

(54) **PHENOLIC ACID ESTERASE AND USE THEREOF**

(57) La présente invention concerne une enzyme présentant une activité d'estérase d'acide phénolique, un gène codant cette enzyme ainsi qu'un procédé pour la production et l'utilisation de cette enzyme.

(57) The present invention relates to an enzyme with phenolic acid esterase activity, gene encoding said enzyme as well as a method for the production and use of said enzyme.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/55, 9/18, C12P 7/42, 19/00, D21H 17/00, A23K 1/165 // (C12N 9/18, C12R 1:645)

A3

(11) International Publication Number: **WO 98/46768**

(43) International Publication Date: 22 October 1998 (22.10.98)

(21) International Application Number: PCT/EP98/02080

(22) International Filing Date: 9 April 1998 (09.04.98)

(30) Priority Data:
9707540.2 14 April 1997 (14.04.97) GB

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(88) Date of publication of the international search report:
21 January 1999 (21.01.99)

(54) Title: PHENOLIC ACID ESTERASE AND USE THEREOF

(57) Abstract

The present invention relates to an enzyme with phenolic acid esterase activity, gene encoding said enzyme as well as a method for the production and use of said enzyme.

International Application PCT/EP98/02080
The Babraham Institute et al

73069 m2/sr
2 June 1999

Proposed Claims:

1. Enzyme with phenolic acid esterase activity including ferulic acid esterase activity and coumaric acid esterase activity, characterized in that said enzyme has a pH optimum greater than pH 6.5 and a temperature optimum greater than 45°C when measured in a citric acid/disodium hydrogen orthophosphate buffer containing 33 µM FAXX as a substrate as well as a K_m of about 3.0µm and a V_{max} of about 35µmol/min/mg protein when measured at 37°C and pH 6.0 in MOPS buffer containing FAXX as a substrate.

2. Enzyme according to claim 1, characterized in that said enzyme has a pH optimum of about pH 7.0 and/or a temperature optimum of about 55°C.

3. Enzyme according to any of claims 1 or 2, characterized in that said enzyme is obtainable from Piromyces Sp., preferably Piromyces equi deposited at the International Mycological Institute (IMI) under the accession number 375061.

4. Enzyme according to any of claims 1 to 3, characterized in that said enzyme comprises the amino acid sequence given in SEQ ID NO:1 or SEQ ID NO:3 or functional derivatives thereof.

5. Enzyme according to any of claims 1 to 4, characterized in that said enzyme is encoded by the DNA sequence given in SEQ ID NO:1 or SEQ ID NO:3 or functional derivatives or homologues thereof.

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6. DNA molecule encoding an enzyme according to any of claims 1 to 5, characterized in that said DNA molecule comprises a DNA sequence as given in SEQ ID NO:1 or SEQ ID NO:3 or functional derivatives or homologues thereof.

7. DNA molecule according to claim 6 further comprising vector sequence capable of expressing said enzyme in a procaryotic or eucaryotic host.

8. Transformed procaryotic cell or eucaryotic cell or organism comprising one or more DNA molecules according to claim 6 or 7.

9. Method for the production of an enzyme or enzyme preparation having phenolic acid esterase activity according to any of claims 1 to 5, characterized in that said enzyme is isolated from a cell or organism according to claim 8.

10. Enzyme preparation comprising enzyme according to any of claims 1 to 5 and/or obtainable by the method according to claim 9.

11. Enzyme preparation according to claim 10 comprising one or more further polysaccharide modifying and/or degrading enzymes.

12. Enzyme preparation according to claim 11, characterized in that said polysaccharide modifying and/or degrading enzyme is selected from the group comprising xylanase, arabinanase, α-L-arabinofuranosidase, endoglucanase, α-D-glucuronidase, pectinase, acetyl esterase, mannanase, acetyl xylan esterase and other glycosyl hydrolases.

13. Enzyme preparation according to any of claims 10 to 12 comprising one or more further enzymes selected from the group comprising amylase, protease, α-galactosidase, phytase and lipase.

14. Use of the enzyme according to any of claims 1 to 5 and/or enzyme preparation according to any of claims 10 to 13 in a process for releasing or preparing phenolic acids from a substrate comprising phenolic acid moieties.

15. Use of the enzyme according to any of claims 1 to 5 and/or enzyme preparation according to any of claims 10 to 13 in the production of animal feed.

16. Use of the enzyme according to any of claims 1 to 5 and/or enzyme preparation according to any of claims 10 to 13 in the production of food.

17. Use of the enzyme according to any of claims 1 to 5 and/or enzyme preparation according to any of claims 10 to 13 in the production of paper.

18. Use of the enzyme according to any of claims 1 to 5 and/or enzyme preparation according to any of claims 10 to 13 in a process for bioconversion of plant material or ligno-cellulose wastes to sugars.

19. Use of the enzyme according to any of claims 1 to 5 and/or enzyme preparation according to any of claims 10 to 13 in crop plants to improve the digestibility of said plants for livestock.

20. Feed additive comprising the enzyme according to any of claims 1 to 5 and/or enzyme preparation according to any of claims 10 to 13.

21. Feed comprising the feed additive according to claim 20.

Phenolic Acid Esterase and Use Thereof

The present invention relates to an enzyme with phenolic acid esterase activity, DNA molecule encoding said enzyme as well as a method for the production and use of said enzyme.

Background

Plant cell walls are divided into two sections, the primary and secondary cell wall. The primary cell wall is comprised of three major classes of polysaccharides: cellulose, hemicellulose and pectin. The secondary cell wall also contains polysaccharides as well as lignin. Hemicellulose, a general class of highly branched polysaccharides found in the plant cell wall, is bound to itself as well as to cellulose and lignin, and these bonds contribute to the stability and support of the plant structure.

Hemicelluloses based on a xylose backbone are designated as xylans. Xylan, which has been shown to exist in a wide variety of different plants including fruits, vegetables legumes, cereals, grasses, softwoods and hardwoods, is a linear β-(1-4)-D-xylopyranose polymer which can be substituted with sugar residues, including α-L-arabinose, and α-D-glucuronic acid and/or the 4-O-methyl ether derivative of α-D-glucuronic acid. Many xylans are also esterified with phenolic acid residues, including coumaric acid and ferulic acid. These phenolic acid residues are present in an ester linkage to α-L-arabinofuranosyl xylan and can serve to protect xylan from xylan-degrading enzymes, so-called xylanases, as well they confer structural stability to the plant cell wall by forming covalent bonds with the lignin present therein. In addition, ferulic acid has been shown to exist as a diferulic acid bridge between different xylan chains, imparting further structural support for plant cells (Linden, J.C. et al., American Chemical Society Symposium Series, vol. 566 (1994), 452-467).

A number of microorganisms are known which are capable of hydrolysing phenolic acid esters and digesting plant cell walls through the enzymatic breakdown of plant cell wall polysaccharides. Some of these microorganisms possess enzyme(s) with phenolic acid esterase activity, i.e. coumaric acid esterase activity or ferulic acid esterase activity or a combination of these two activities.

For example, Borneman, W.S. et al (Applied and Environmental Microbiology, vol. 58 (1992), 3762-3766) describe two ferulic acid esterases (FAE), designated FAE-I and FAE-II respectively, isolated from the anaerobic fungus Neocallimastix strain MC-2. FAE-II was reported to be specific for the substrate (O-{5-O-[(E)-feruloyl]-α-L-arabinofuranosyl}-(1-3)-O-β-D-xylopyranosyl-(1-4)-D-xylopyranose (FAXX), whereas FAE-I was reported to have both a FAXX degrading activity as well as a (O-{5-O-[(E)-p-coumaroyl]-α-L-arabinofuranosyl}-(1-3)-O-β-D-xylopyranosyl-(1-4)-D-xylopyranose (PAXX) degrading activity, the maximum ratio of metabolism of FAXX:PAXX being 3:1. The pH optima of these two enzymes were shown to be 6.2 and 7.0 respectively when using FAXX as a substrate.

GB 2 301 103 discloses an FAE obtained from Aspergillus niger as well as the gene encoding said enzyme. Said enzyme has a pH optimum of about 5 and a temperature optimum of from about 50 to 60°C when methyl ferulate is used as a substrate.

Other purified enzymes with ferulic acid esterase activity are known (for example, see McCrae, S.I. et al., Enzyme Microb. Technol., vol. 16 (1994), 826-834; Faulds, C.B. and Williamson, G., Microbiology, vol. 140 (1994), 779-787; Castanares, A. et al., Enzyme Microb. Technol., vol. 14 (1992), 875; and Kroon, P.A. et al., Biotechnol. Appl. Biochem., vol 23 (1996), 255-262) which have pH optima ranging from about 5.0 to 6.0 and temperature optima from 30 to 60°C.

Enzymes with phenolic acid esterase activity can be used in a number of industrial, agricultural and health applications which can be carried out at pH values about or above 6.5 and/or at temperatures above 45°C.

Summary of the invention

It is an object of the present invention to provide enzyme with good phenolic acid esterase activity.

In addition, it is an object of the present invention to provide a source of an enzyme with phenolic acid esterase activity which is available in relatively large amounts.

Furthermore, it is an object to provide a method for the production of an enzyme with phenolic acid esterase activity.

A further object is to provide uses of an enzyme phenolic acid esterase activity for the preparation of food and feed, for the processing of paper and pulp as well as for the bioconversion of ligno-cellulose wastes, for example.

Other objects of the present invention will become apparent from the following detailed description.

Subject matter of the present invention is an enzyme with phenolic acid esterase activity, characterized in that said enzyme has a pH optimum greater than pH 6.5, preferably about 7.0, and a temperature optimum greater than 45°C, preferably greater than 50°C, and more preferably about 55°C, when measured in a citric acid/disodium hydrogen orthophosphate buffer containing 33 μM FAXX as a substrate. Preferably, said enzyme has ferulic acid esterase activity and coumaric acid esterase activity.

Subject matter of the present invention is also an enzyme with phenolic acid esterase activity obtainable from Piromyces Sp., for example Piromyces equi, and more preferably from the Piromyces equi strain deposited under the Budapest Treaty at the International Mycological Institute (IMI), Bakeham Lane, Egham, Surrey, UK under the Accession Number 375061.

Preferably, the enzyme of the present invention comprises the amino acid sequence given in SEQ ID NO:1 or a functional derivative thereof. A functional derivative of the enzyme of the present invention is defined as an enzyme having one or more N-terminal, C-terminal or internal substitution(s), insertion(s) and/or deletion(s) in the amino acid sequence given in SEQ ID NO:1 which maintains a pH optimum greater than 6.5, preferably about 7.0, and a temperature optimum greater than 45°C, preferably greater than 50°C, and more preferably about 55°C, when measured in a citric acid/disodium hydrogen orthophosphate buffer containing 33 μM FAXX as a substrate. More preferably, the enzyme of the present invention comprises the amino acid sequence given in SEQ ID NO:3 or a functional derivative thereof.

In addition, the enzyme of the present invention is preferably encoded by the DNA sequence given in SEQ ID NO:1 or a functional derivative thereof. More preferably, the enzyme of the present invention is encoded by the DNA sequence given in SEQ ID NO:3 or a functional derivative thereof.

The present invention relates to a phenolic acid esterase with one or more of the above properties.

Further subject matter of the present invention is a DNA molecule encoding an enzyme with phenolic acid esterase activity, characterized in that said DNA molecule comprises a DNA sequence as given in SEQ ID NO:1 or a functional derivative or homologue thereof. A functional derivative of the DNA sequence given in SEQ ID NO:1 is defined as a DNA

sequence having one or more 5'-, 3'- or internal substitution(s), insertion(s) and/or deletion(s) in the DNA sequence given in SEQ ID NO:1 which maintains its capability to encode an enzyme with phenolic acid esterase activity which has a pH optimum greater than 6.5, preferably about 7.0, and a temperature optimum greater than 45°C, preferably greater than 50°C, and more preferably about 55°C, when measured in a citric acid/disodium hydrogen orthophosphate buffer comprising 33 μM FAXX as a substrate. A functional homologue of the DNA sequence of the present invention is defined as a DNA sequence with preferably 75% homology, more preferably 85% homology and most preferably 95% homology to the DNA sequence given in SEQ ID NO:1 or SEQ ID NO:3. More preferably, a DNA molecule encoding an enzyme according to the present invention comprises a DNA sequence as given in SEQ ID NO:3 or a functional derivative or homologue thereof.

In a preferred embodiment, DNA molecules of the present invention comprise vector sequence capable of replicating said DNA molecules and/or expressing said enzyme in a procaryotic or eucaryotic host.

Further subject matter of the present invention is a transformed procaryotic cell or eucaryotic cell comprising one or more DNA molecules of the present invention. Preferably said cells are selected from the group comprising E. coli, Bacillus sp., such as Bacillus subtilis, Lactobacillus sp., and Lactococcus sp., Aspergillus, Trichoderma, Penicillium, Mucor, Kluyveromyces and Saccharomyces, such as Saccharomyces cerevisiae.

The enzyme of the present invention may be expressed in transgenic plants such as maize, soybean and canola/rapeseed. or in root storage organs of plants, such as potato, carrot and sugar beet.

The introduction of an esterase of the present invention expressed and/or secreted at the appropriate stage, for example, at harvest, has the advantage that the risk of weakening the transgenic plant or storage root organ structure during growth can be reduced.

The methodology for the production of transformed procaryotic and eucaryotic cells is known in the art. Transgenic fungus, such as Aspergillus, tranformed yeast, such as Saccharomyces, and transgenic plants are also known inthe art and can be produced by the methods taught and discussed in GB 2 301 103, EP 479 359 and EP 449 375.

Subject matter of the present invention is also a method for the production of an enzyme or enzyme preparation having phenolic acid esterase activity, characterized in that said enzyme is isolated from a naturally occurring organism or transformed cell or organism capable of expressing the enzyme according to the invention. Enzyme preparations including, for example, partially purified preparations obtainable as a cell or organism extract are also subject matter of the present invention.

The enzyme preparation of the present invention can comprise one or more further polysaccharide modifying and/or degrading enzymes. Said polysaccharide modifying and/or degrading enzyme(s) is (are) preferably selected from the group comprising xylanase, arabinanase, α-L-arabinofuranosidase, endoglucanase, α-D-glucuronidase, pectinase, acetyl esterase, mannanase, acetyl xylan esterase and other glycosyl hydrolases.

In addition, the enzyme preparation of the present invention can also include one or more further enzymes selected from the group comprising amylase, protease, α-galactosidase, phytase and lipase.

Use of the enzyme and/or enzyme preparation according to the invention include the use in a process for releasing phenolic acids from a substrate comprising phenolic acid moieties.

Said enzyme and/or enzyme preparation according to the invention can equally find use in the production of animal feed by improving the digestibility of plant material, especially forage in which the plant cell walls have a high phenolic acid content. Furthermore, the enzyme and/or the enzyme preparation according to the invention can be used in or with crop plants including but not limited to maize, wheat, grasses and alfalfa, to improve the digestability for livestock by pre-modifying the cell wall content. Said enzyme and/or enzyme preparation according to the invention can also find used in the preparation of food for human consumption.

Further subject matter of the present invention is also a feed additive comprising an enzyme or enzyme preparation having phenolic acid esterase activity according to the invention and a feed comprising said feed additive.

The enzyme and/or enzyme preparation according to the invention can also find use in the paper and pulp industry, for example, in helping remove lignin from cellulose pulps. Additionally, used in combination with xylan degrading enzymes, the enzyme and/or enzyme preparation according to the invention can contribute to a reduction in the amount of chlorine required for bleaching by increasing the solubility and extractability of lignin from pulp.

Furthermore, when combined with xylanases and/or cellulases, the enzyme and/or enzyme preparation according to the invention can be used for the bioconversion of plant material or ligno-cellulose wastes to sugars, for example, for chemical or fuel production, and/or in the production of phenolic acids.

Brief description of the drawings

Figure 1: pH profile of the phenolic acid esterase of the present invention measured using FAXX as a substrate.

Figure 2: Temperature profile of the phenolic acid esterase of the present invention measured using FAXX as a substrate.

Detailed description of the invention

The following Examples are intended to more closely illustrate the present invention without limiting the subject matter of the invention to said Examples.

Example 1

Piromyces equi isolated from horse cecum (Orpin, C.G., J. Gen. Microbiol., vol. 123 (1981), 287-296) and as described by E.A. Munn in Anaerobic Fungi, Biology, Ecology and Function, D.O. Mountfort and C.G. Orpin Eds., Marcel Dekker, Inc., New York, 1994, 47-105, and deposited under the Budapest Treaty at the International Mycological Institute (IMI) under the Accession Number 375061 was cultured under anaerobic conditions at a temperature of 39°C in a rumen fluid-containing medium (Kemp,P., Lander, D.J. and Orpin, C.G., J. Gen. Microbiol., vol. 130 (1984), 27-37) with 0.10% soluble xylan and 0.5% Sigmacell (Sigma Chemical Co., Poole, Dorset, England) as carbon sources. Total RNA was extracted from fungus grown under the above conditions, poly(A)+ RNA was selected by oligo(dT) chromatography, and double-stranded cDNA was synthesized from the selected RNA, cloned into λZAPII using a ZAP-cDNA synthesis kit and packaged in vitro according to the instructions of the manufacturer (Stratagene, La Jolla, California, USA) (Xue, G-P. et al., J Gen. Microbiol., vol. 138 (1992), 1413-1420 and Ali, B.R.S. et al., FEMS Microbiol.

Lett., vol. 125 (1995), 15-22). Recombinant phage were grown by plating on lawns of E. coli XL1-Blue in soft agar overlays and screened using an antibody raised against a fungal cellulase/hemicellulase complex purified according to Ali,B.R.S. et al., FEMS Microbiol. Lett., vol. 125 (1995), 15-22). Antibody screening of phage plaques with rabbit anti-complex antibody as the primary antibody was carried out essentially as described in the instruction manual provided with the picoBlue™ immunoscreening kit (Stratagene), with the following modifications: isopropyl-β-D-thiogalactopyranoside (IPTG: 0.33 mM) was added directly to the soft agar overlays containing recombinant λZAPII and host bacteria (E. coli XL1-Blue); plaques were lifted onto Hybond-C filters (Amersham); blocking solution contained dried milk powder (4% w/v) in place of BSA; anti-rabbit IgG (whole molecule) conjugated to horseradish peroxidase (Sigma Chemical Co.) was used as secondary antibody; colour development solution comprised 3,3'-diaminobenzidine (0.5 mg/ml) in 50 mM Tris-HCl buffer, pH 7.4, containing hydrogen peroxide (0.5 µl/ml. Esterase production was verified by showing that a clone selected by antibody screening synthesized an enzyme which hydrolysed [4-methylumbelliferoyl(p-trimethylammonium cinnamate chloride)] according to Dalrymple, B.P. et al., FEMS Microbiol. Lett., vol 143 (1996), 115-120.

General molecular biological techniques including DNA isolation, restriction endonuclease digestion, ligation, transformation as well as DNA sequencing of the esterase gene were performed in accordance with Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd. Ed. (1989), Cold Spring Harbor, New York.

Nucleotide sequencing of the the gene encoding the enzyme having phenolic acid esterase activity of the present invention was performed and the results are given in SEQ ID NO:3. The open reading frame comprises 1608 nucleotides,

encoding a protein of 536 amino acids with a predicted molecular weight of 55,540 daltons.

Example 2

Measurement of pH optimum

A truncated enzyme encoded by SEQ ID NO. 1 was generated in a PCR reaction (20 cycles of 30 seconds at 94°C, 45 seconds at 50°C, and 1 minute at 72°C) in a buffer comprising 50 mM Tris-HCl, pH 9.0, 50 mM NaCl, 10 mM $MgCl_2$, 200 µM dNTPs, 50 picomoles of the primers
5'-end: 5'-CGCGGATCCAACAGCGGTCCAACTGTTG-3'
3'-end: 5'-GCGAATTCTTATCTTATGGGAGAGAG-3', and
250 ng template DNA and expressed in E.coli BL21 (DE3) (Novagen, Inc., Wisconsin, USA) using the vector pET32a (Novagen, Inc.).

The enzyme was purified from freshly prepared cell-free extracts by binding to Talon resin (Clontech Laboratories Inc., California, USA) and cleaved from the metal affinity resin using restriction grade Thrombin (Sigma) in accordance to the guidelines provided by Novagen, Inc., USA, for use with pET vectors. The enzyme was further purified as follows: a 1 ml MonoQ column (Pharmacia) was equilibrated with 10 mM Tris, pH 8.0, and fresh enzyme was applied. The enzyme was eluted at 1.0 ml/min with a sodium chloride gradient (0 to 0.5 M NaCl in 10 mM Tris, pH 8.0). Fractions of 1.0 ml were collected. The enzyme was assayed in McIlvaine's buffer (citric acid/ disodium hydrogen orthophosphate, see Data for Biochemical Research, 3rd Edition, Dawson, Elliot, Elliot, Jones, Oxford Science Publications, Oxford University Press, 1987) for pH values ranging from 3 to 7 or a buffer comprising potassium chloride/ boric acid for pH values ranging from 8 to 9. The assay was carried out at 37°C with a final FAXX concentration of 33 µM. Ferulic acid release from FAXX was monitored

continuously for 3 min at 335 nm according to (Faulds, C.B. and Williamson, G., Microbiology, vol. 140 (1994), 779-787).

The results of the assay are given in Figure 1. As can be deduced from Figure 1, the enzyme of the invention exhibited 50% activity at about pH 5.5 and about 8.5

In order to determine the temperature optimum of the enzyme according to the invention using FAXX as a substrate, FAXX was employed at a concentration of 33 μM and the assay was performed at pH 6.0 in 100 mM MOPS buffer. The temperature of incubation was changed from 20°C to 70°C using a thermostatically controlled spectrophotometer. The release of ferulic acid from FAXX was measured at 335 nm as described above. The results are presented in Figure 2.

Kinetics

The K_m and V_{max} of the enzyme of the present invention were determined using FAXX and Ara_2F (O-[2-O(trans-feruloyl)-α-arabinofuranosyl]-(1-5)-L-arabinofuranose) as substrates. FAXX was employed at concentrations varying from 3.72 μM to 49.18 μM and Ara_2F was used at concentrations ranging from 4.46 μM to 122.92 μM. The assay was performed at 37°C and pH 6.0 in 100 mM MOPS ((3-[N-morpholino]propanesulfonic acid)) buffer with 90 ng enzyme. For both substrates, the release of ferulic acid was measured at 335 nM as described above.

Based on results of the above experiments, it was determined that the enzyme of the present invention has the following kinetic constants:

substrate	K_m	V_{max}
FAXX	3.0±0.3 μM	35.6±0.9 μmol/min/mg
Ara_2F	234±27 μM	19.6±1.7 μmol/min/mg.

Hence, the enzyme of the present invention has a K_m of about 3.0 and a V_{max} of about 35 when measured under the above conditions using FAXX as a substrate.

The specific activity of the enzyme of the present invention was determined for methyl ferulate, methyl coumarate and methyl p-coumarate in an assay at 37°C comprising 100 mM MOPS buffer (with 0.02% azide), pH 6.0., 44 ng enzyme and 1 mM of the above substrates. After 15 minutes incubation time, the reaction was terminated by boiling and the free acid liberated was measured using reverse phase HPLC (Kroon, P.A. and Williamson, G., Biotechnol. Appl. Biochem., vol. 23 (1996), 263-267). The results of the above experiment are shown below.

In addition, the specific activity of the enzyme of the present invention was determined for p-nitrophenyl acetate, α-naphthyl acetate, α-naphthyl butyrate, α-naphthyl caproate α-naphthyl caprylate and α-naphthyl laurate according to the methods described in Ferreira, L.M.A. et al.(Biochem. J., vol. 294 (1993), 349-355). The results of the above assay are shown below.

substrate	specific activity (U*/mg)
p-nitrophenyl acetate	204.3
α-naphthyl acetate	121
α-naphthyl butyrate	220
α-naphthyl caproate	256
α-naphthyl caprylate	54
α-naphthyl laurate	6
methyl ferulate	10.6
methyl coumarate	10.5
methyl p-coumarate	2.7

*1 U is defined as the amount of enzyme which gives 1 μmol/min of ester hydrolysis.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Biotechnology and Biological Sciences Research Council
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(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): SN2 1UH

(ii) TITLE OF INVENTION: Phenolic Acid Esterase and Use Thereof

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 825 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Piromyces equi

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:1..822

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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AAC AGC GGT CCA ACT GTT GAA TAC TCT ACT GAT GTT GAC TGT TCC GGT      48
Asn Ser Gly Pro Thr Val Glu Tyr Ser Thr Asp Val Asp Cys Ser Gly
 1               5                  10                  15

AAG ACC CTT AAG AGT AAC ACC AAC CTT AAC ATC AAT GGT CGT AAG GTT      96
Lys Thr Leu Lys Ser Asn Thr Asn Leu Asn Ile Asn Gly Arg Lys Val
            20                  25                  30

ATT GTA AAA TTC CCA AGC GGC TTC ACT GGT GAC AAG GCT GCT CCA CTT     144
Ile Val Lys Phe Pro Ser Gly Phe Thr Gly Asp Lys Ala Ala Pro Leu
        35                  40                  45

CTT ATT AAC TAC CAT CCA ATT ATG GGT AGT GCT TCT CAA TGG GAA AGT     192
Leu Ile Asn Tyr His Pro Ile Met Gly Ser Ala Ser Gln Trp Glu Ser
    50                  55                  60

GGT TCT CAA ACT GCT AAG GCT GCT TTA AAT GAT GGT GCC ATC GTT GCT     240
Gly Ser Gln Thr Ala Lys Ala Ala Leu Asn Asp Gly Ala Ile Val Ala
65                  70                  75                  80

TTC ATG GAT GGT GCT CAA GGT CCA ATG GGA CAA GCT TGG AAC GTT GGT     288
Phe Met Asp Gly Ala Gln Gly Pro Met Gly Gln Ala Trp Asn Val Gly
                85                  90                  95

CCA TGT TGT ACT GAT GCT GAT GAT GTT CAA TTC ACT CGT AAC TTC ATT     336
Pro Cys Cys Thr Asp Ala Asp Asp Val Gln Phe Thr Arg Asn Phe Ile
            100                 105                 110

AAG GAA ATC ACT AGT AAG GCT TGT GTT GAT CCA AAG CGT ATC TAT GCT     384
Lys Glu Ile Thr Ser Lys Ala Cys Val Asp Pro Lys Arg Ile Tyr Ala
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115 120 125

GCT GGT TTC TCT ATG GGT GGT GGT ATG TCT AAC TAT GCT GGT TGT CAA 432
Ala Gly Phe Ser Met Gly Gly Gly Met Ser Asn Tyr Ala Gly Cys Gln
130 135 140

CTT GCT GAT GTT ATT GCT GCT GCT GCT CCA TCA GCC TTT GAT CTT GCC 480
Leu Ala Asp Val Ile Ala Ala Ala Ala Pro Ser Ala Phe Asp Leu Ala
145 150 155 160

AAG GAA ATT GTT GAT GGT GGT AAA TGT AAA CCA GCT CGT CCA TTC CCA 528
Lys Glu Ile Val Asp Gly Gly Lys Cys Lys Pro Ala Arg Pro Phe Pro
165 170 175

ATC CTT AAC TTC CGT GGT ACT CAA GAT AAC GTT GTT ATG TAC AAC GGT 576
Ile Leu Asn Phe Arg Gly Thr Gln Asp Asn Val Val Met Tyr Asn Gly
180 185 190

GGT CTT TCT CAA GTT GTT CAA GGT AAG CCA ATT ACT TTC ATG GGT GCC 624
Gly Leu Ser Gln Val Val Gln Gly Lys Pro Ile Thr Phe Met Gly Ala
195 200 205

AAG AAC AAC TTC AAG GAA TGG GCT AAG ATG AAC GGA TGT ACT GGT GAA 672
Lys Asn Asn Phe Lys Glu Trp Ala Lys Met Asn Gly Cys Thr Gly Glu
210 215 220

CCA AAA CAA AAC ACT CCA GGT AAC AAC TGT GAA ATG TAC GAA AAC TGT 720
Pro Lys Gln Asn Thr Pro Gly Asn Asn Cys Glu Met Tyr Glu Asn Cys
225 230 235 240

AAG GGT GGT GTT AAG GTT GGT CTT TGC ACT ATC AAC GGT GGT GGT CAC 768
Lys Gly Gly Val Lys Val Gly Leu Cys Thr Ile Asn Gly Gly Gly His
245 250 255

GCT GAA GGT GAC GGT AAA ATG GGT TGG GAC TTT GTT AAA CAA TTC TCT 816
Ala Glu Gly Asp Gly Lys Met Gly Trp Asp Phe Val Lys Gln Phe Ser
260 265 270

CTC CCA TAA 825
Leu Pro

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 274 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Asn Ser Gly Pro Thr Val Glu Tyr Ser Thr Asp Val Asp Cys Ser Gly
1 5 10 15

Lys Thr Leu Lys Ser Asn Thr Asn Leu Asn Ile Asn Gly Arg Lys Val
20 25 30

Ile Val Lys Phe Pro Ser Gly Phe Thr Gly Asp Lys Ala Ala Pro Leu
35 40 45

Leu Ile Asn Tyr His Pro Ile Met Gly Ser Ala Ser Gln Trp Glu Ser
50 55 60

Gly Ser Gln Thr Ala Lys Ala Ala Leu Asn Asp Gly Ala Ile Val Ala
65 70 75 80

Phe Met Asp Gly Ala Gln Gly Pro Met Gly Gln Ala Trp Asn Val Gly
85 90 95

Pro Cys Cys Thr Asp Ala Asp Asp Val Gln Phe Thr Arg Asn Phe Ile
100 105 110

Lys Glu Ile Thr Ser Lys Ala Cys Val Asp Pro Lys Arg Ile Tyr Ala
115 120 125

Ala Gly Phe Ser Met Gly Gly Gly Met Ser Asn Tyr Ala Gly Cys Gln
130 135 140

Leu Ala Asp Val Ile Ala Ala Ala Ala Pro Ser Ala Phe Asp Leu Ala
145 150 155 160

Lys Glu Ile Val Asp Gly Gly Lys Cys Lys Pro Ala Arg Pro Phe Pro
165 170 175

Ile Leu Asn Phe Arg Gly Thr Gln Asp Asn Val Val Met Tyr Asn Gly
180 185 190

Gly Leu Ser Gln Val Val Gln Gly Lys Pro Ile Thr Phe Met Gly Ala
195 200 205

Lys Asn Asn Phe Lys Glu Trp Ala Lys Met Asn Gly Cys Thr Gly Glu
210 215 220

Pro Lys Gln Asn Thr Pro Gly Asn Asn Cys Glu Met Tyr Glu Asn Cys
225 230 235 240

Lys Gly Gly Val Lys Val Gly Leu Cys Thr Ile Asn Gly Gly Gly His
245 250 255

Ala Glu Gly Asp Gly Lys Met Gly Trp Asp Phe Val Lys Gln Phe Ser
260 265 270

Leu Pro

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1611 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Piromyces equi

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:1..1608

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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ATG AAG ACT AGC ATT GTA TTA TCT ATC GTT GCT TTA TTT TTA ACA TCC      48
Met Lys Thr Ser Ile Val Leu Ser Ile Val Ala Leu Phe Leu Thr Ser
275                 280                 285                 290

AAA GCT TCT GCT GAT TGT TGG TCA GAA AGA TTA GGT TGG CCA TGC TGT      96
Lys Ala Ser Ala Asp Cys Trp Ser Glu Arg Leu Gly Trp Pro Cys Cys
                295                 300                 305

AGT GAC AGC AAT GCC GAA GTA ATC TAC GTC GAT GAC GAT GGT GAT TGG     144
Ser Asp Ser Asn Ala Glu Val Ile Tyr Val Asp Asp Asp Gly Asp Trp
            310                 315                 320

GGT GTT GAA AAT AAT GAC TGG TGT GGT ATC CAA AAG GAA GAA GAA AAC     192
Gly Val Glu Asn Asn Asp Trp Cys Gly Ile Gln Lys Glu Glu Glu Asn
        325                 330                 335

AAT AAC TCA TGG GAT ATG GGT GAT TGG AAC CAA GGT GGT AAC CAA GGT     240
Asn Asn Ser Trp Asp Met Gly Asp Trp Asn Gln Gly Gly Asn Gln Gly
    340                 345                 350

GGC GGT ATG CCA TGG GGC GAC TTT GGC GGT AAC CAA GGT GGT GGT ATG     288
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Gly Gly Met Pro Trp Gly Asp Phe Gly Gly Asn Gln Gly Gly Gly Met
355 360 365 370

CAA TGG GGT GAC TTC GGT GGT AAC CAA GGT GGC GGT ATG CCA TGG GGC 336
Gln Trp Gly Asp Phe Gly Gly Asn Gln Gly Gly Gly Met Pro Trp Gly
375 380 385

GAC TTC GGT GGT AAC CAA GGT GGC GGT ATG CCA TGG GGT GAC TTT GGC 384
Asp Phe Gly Gly Asn Gln Gly Gly Gly Met Pro Trp Gly Asp Phe Gly
390 395 400

GGT AAC CAA GGT GGT AAC CAA GGC GGT GGT ATG CCA TGG GGC GAC TTT 432
Gly Asn Gln Gly Gly Asn Gln Gly Gly Gly Met Pro Trp Gly Asp Phe
405 410 415

GGA GGA AAC CAA GGA GGT AAC CAA GGT GGC GGT ATG CCA TGG GGT GAT 480
Gly Gly Asn Gln Gly Gly Asn Gln Gly Gly Gly Met Pro Trp Gly Asp
420 425 430

TTC GGA GGT AAC CAA GGT GGT GGT ATG CAA TGG GGC GAC TTT GGA GGA 528
Phe Gly Gly Asn Gln Gly Gly Gly Met Gln Trp Gly Asp Phe Gly Gly
435 440 445 450

AAC CAA GGA GGT AAC CAA GGT GGC GGT ATG CCA TGG GGT GAT TTC GGA 576
Asn Gln Gly Gly Asn Gln Gly Gly Gly Met Pro Trp Gly Asp Phe Gly
455 460 465

GGT AAC CAA GGT GGT GGT ATG CAA TGG GGC GAC TTT GGA GGA AAC CAA 624
Gly Asn Gln Gly Gly Gly Met Gln Trp Gly Asp Phe Gly Gly Asn Gln
470 475 480

GGA GGT AAC CAA GGT GGC GGT ATG CCA TGG GGT GAC TTC GGA GGT AAC 672
Gly Gly Asn Gln Gly Gly Gly Met Pro Trp Gly Asp Phe Gly Gly Asn
485 490 495

CAA GGT GGT GGT ATG CAA TGG GGC GAT TTC GGA GGT AAT CAA GGT GGT 720
Gln Gly Gly Gly Met Gln Trp Gly Asp Phe Gly Gly Asn Gln Gly Gly

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500                 505                 510

GGT ATG CAA TGG GGC GAC TTC GGC GGT AAC CAA GGA GGT AAC CAA GAT      768
Gly Met Gln Trp Gly Asp Phe Gly Gly Asn Gln Gly Gly Asn Gln Asp
515                 520                 525                 530

TGG GGT AAC CAA GGT GGT AAC AGC GGT CCA ACT GTT GAA TAC TCT ACT      816
Trp Gly Asn Gln Gly Gly Asn Ser Gly Pro Thr Val Glu Tyr Ser Thr
                535                 540                 545

GAT GTT GAC TGT TCC GGT AAG ACC CTT AAG AGT AAC ACC AAC CTT AAC      864
Asp Val Asp Cys Ser Gly Lys Thr Leu Lys Ser Asn Thr Asn Leu Asn
            550                 555                 560

ATC AAT GGT CGT AAG GTT ATT GTA AAA TTC CCA AGC GGC TTC ACT GGT      912
Ile Asn Gly Arg Lys Val Ile Val Lys Phe Pro Ser Gly Phe Thr Gly
        565                 570                 575

GAC AAG GCT GCT CCA CTT CTT ATT AAC TAC CAT CCA ATT ATG GGT AGT      960
Asp Lys Ala Ala Pro Leu Leu Ile Asn Tyr His Pro Ile Met Gly Ser
    580                 585                 590

GCT TCT CAA TGG GAA AGT GGT TCT CAA ACT GCT AAG GCT GCT TTA AAT     1008
Ala Ser Gln Trp Glu Ser Gly Ser Gln Thr Ala Lys Ala Ala Leu Asn
595                 600                 605                 610

GAT GGT GCC ATC GTT GCT TTC ATG GAT GGT GCT CAA GGT CCA ATG GGA     1056
Asp Gly Ala Ile Val Ala Phe Met Asp Gly Ala Gln Gly Pro Met Gly
                615                 620                 625

CAA GCT TGG AAC GTT GGT CCA TGT TGT ACT GAT GCT GAT GAT GTT CAA     1104
Gln Ala Trp Asn Val Gly Pro Cys Cys Thr Asp Ala Asp Asp Val Gln
            630                 635                 640

TTC ACT CGT AAC TTC ATT AAG GAA ATC ACT AGT AAG GCT TGT GTT GAT     1152
Phe Thr Arg Asn Phe Ile Lys Glu Ile Thr Ser Lys Ala Cys Val Asp
        645                 650                 655
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CCA AAG CGT ATC TAT GCT GCT GGT TTC TCT ATG GGT GGT GGT ATG TCT 1200
Pro Lys Arg Ile Tyr Ala Ala Gly Phe Ser Met Gly Gly Gly Met Ser
660 665 670

AAC TAT GCT GGT TGT CAA CTT GCT GAT GTT ATT GCT GCT GCT GCT CCA 1248
Asn Tyr Ala Gly Cys Gln Leu Ala Asp Val Ile Ala Ala Ala Ala Pro
675 680 685 690

TCA GCC TTT GAT CTT GCC AAG GAA ATT GTT GAT GGT GGT AAA TGT AAA 1296
Ser Ala Phe Asp Leu Ala Lys Glu Ile Val Asp Gly Gly Lys Cys Lys
695 700 705

CCA GCT CGT CCA TTC CCA ATC CTT AAC TTC CGT GGT ACT CAA GAT AAC 1344
Pro Ala Arg Pro Phe Pro Ile Leu Asn Phe Arg Gly Thr Gln Asp Asn
710 715 720

GTT GTT ATG TAC AAC GGT GGT CTT TCT CAA GTT GTT CAA GGT AAG CCA 1392
Val Val Met Tyr Asn Gly Gly Leu Ser Gln Val Val Gln Gly Lys Pro
725 730 735

ATT ACT TTC ATG GGT GCC AAG AAC AAC TTC AAG GAA TGG GCT AAG ATG 1440
Ile Thr Phe Met Gly Ala Lys Asn Asn Phe Lys Glu Trp Ala Lys Met
740 745 750

AAC GGA TGT ACT GGT GAA CCA AAA CAA AAC ACT CCA GGT AAC AAC TGT 1488
Asn Gly Cys Thr Gly Glu Pro Lys Gln Asn Thr Pro Gly Asn Asn Cys
755 760 765 770

GAA ATG TAC GAA AAC TGT AAG GGT GGT GTT AAG GTT GGT CTT TGC ACT 1536
Glu Met Tyr Glu Asn Cys Lys Gly Gly Val Lys Val Gly Leu Cys Thr
775 780 785

ATC AAC GGT GGT GGT CAC GCT GAA GGT GAC GGT AAA ATG GGT TGG GAC 1584
Ile Asn Gly Gly Gly His Ala Glu Gly Asp Gly Lys Met Gly Trp Asp
790 795 800

TTT GTT AAA CAA TTC TCT CTC CCA TAA 1611
Phe Val Lys Gln Phe Ser Leu Pro
805 810

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 536 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Lys Thr Ser Ile Val Leu Ser Ile Val Ala Leu Phe Leu Thr Ser
1 5 10 15

Lys Ala Ser Ala Asp Cys Trp Ser Glu Arg Leu Gly Trp Pro Cys Cys
20 25 30

Ser Asp Ser Asn Ala Glu Val Ile Tyr Val Asp Asp Asp Gly Asp Trp
35 40 45

Gly Val Glu Asn Asn Asp Trp Cys Gly Ile Gln Lys Glu Glu Glu Asn
50 55 60

Asn Asn Ser Trp Asp Met Gly Asp Trp Asn Gln Gly Gly Asn Gln Gly
65 70 75 80

Gly Gly Met Pro Trp Gly Asp Phe Gly Gly Asn Gln Gly Gly Gly Met
85 90 95

Gln Trp Gly Asp Phe Gly Gly Asn Gln Gly Gly Gly Met Pro Trp Gly
100 105 110

Asp Phe Gly Gly Asn Gln Gly Gly Gly Met Pro Trp Gly Asp Phe Gly

115 120 125

Gly Asn Gln Gly Gly Asn Gln Gly Gly Gly Met Pro Trp Gly Asp Phe
130 135 140

Gly Gly Asn Gln Gly Gly Asn Gln Gly Gly Gly Met Pro Trp Gly Asp
145 150 155 160

Phe Gly Gly Asn Gln Gly Gly Gly Met Gln Trp Gly Asp Phe Gly Gly
165 170 175

Asn Gln Gly Gly Asn Gln Gly Gly Gly Met Pro Trp Gly Asp Phe Gly
180 185 190

Gly Asn Gln Gly Gly Gly Met Gln Trp Gly Asp Phe Gly Gly Asn Gln
195 200 205

Gly Gly Asn Gln Gly Gly Gly Met Pro Trp Gly Asp Phe Gly Gly Asn
210 215 220

Gln Gly Gly Gly Met Gln Trp Gly Asp Phe Gly Gly Asn Gln Gly Gly
225 230 235 240

Gly Met Gln Trp Gly Asp Phe Gly Gly Asn Gln Gly Gly Asn Gln Asp
245 250 255

Trp Gly Asn Gln Gly Gly Asn Ser Gly Pro Thr Val Glu Tyr Ser Thr
260 265 270

Asp Val Asp Cys Ser Gly Lys Thr Leu Lys Ser Asn Thr Asn Leu Asn
275 280 285

Ile Asn Gly Arg Lys Val Ile Val Lys Phe Pro Ser Gly Phe Thr Gly
290 295 300

Asp Lys Ala Ala Pro Leu Leu Ile Asn Tyr His Pro Ile Met Gly Ser
305 310 315 320

Ala Ser Gln Trp Glu Ser Gly Ser Gln Thr Ala Lys Ala Ala Leu Asn
325 330 335

Asp Gly Ala Ile Val Ala Phe Met Asp Gly Ala Gln Gly Pro Met Gly
340 345 350

Gln Ala Trp Asn Val Gly Pro Cys Cys Thr Asp Ala Asp Asp Val Gln
355 360 365

Phe Thr Arg Asn Phe Ile Lys Glu Ile Thr Ser Lys Ala Cys Val Asp
370 375 380

Pro Lys Arg Ile Tyr Ala Ala Gly Phe Ser Met Gly Gly Gly Met Ser
385 390 395 400

Asn Tyr Ala Gly Cys Gln Leu Ala Asp Val Ile Ala Ala Ala Ala Pro
405 410 415

Ser Ala Phe Asp Leu Ala Lys Glu Ile Val Asp Gly Gly Lys Cys Lys
420 425 430

Pro Ala Arg Pro Phe Pro Ile Leu Asn Phe Arg Gly Thr Gln Asp Asn
435 440 445

Val Val Met Tyr Asn Gly Gly Leu Ser Gln Val Val Gln Gly Lys Pro
450 455 460

Ile Thr Phe Met Gly Ala Lys Asn Asn Phe Lys Glu Trp Ala Lys Met
465 470 475 480

Asn Gly Cys Thr Gly Glu Pro Lys Gln Asn Thr Pro Gly Asn Asn Cys
485 490 495

Glu Met Tyr Glu Asn Cys Lys Gly Gly Val Lys Val Gly Leu Cys Thr
500 505 510

Ile Asn Gly Gly Gly His Ala Glu Gly Asp Gly Lys Met Gly Trp Asp
515 520 525

Phe Val Lys Gln Phe Ser Leu Pro
530 535

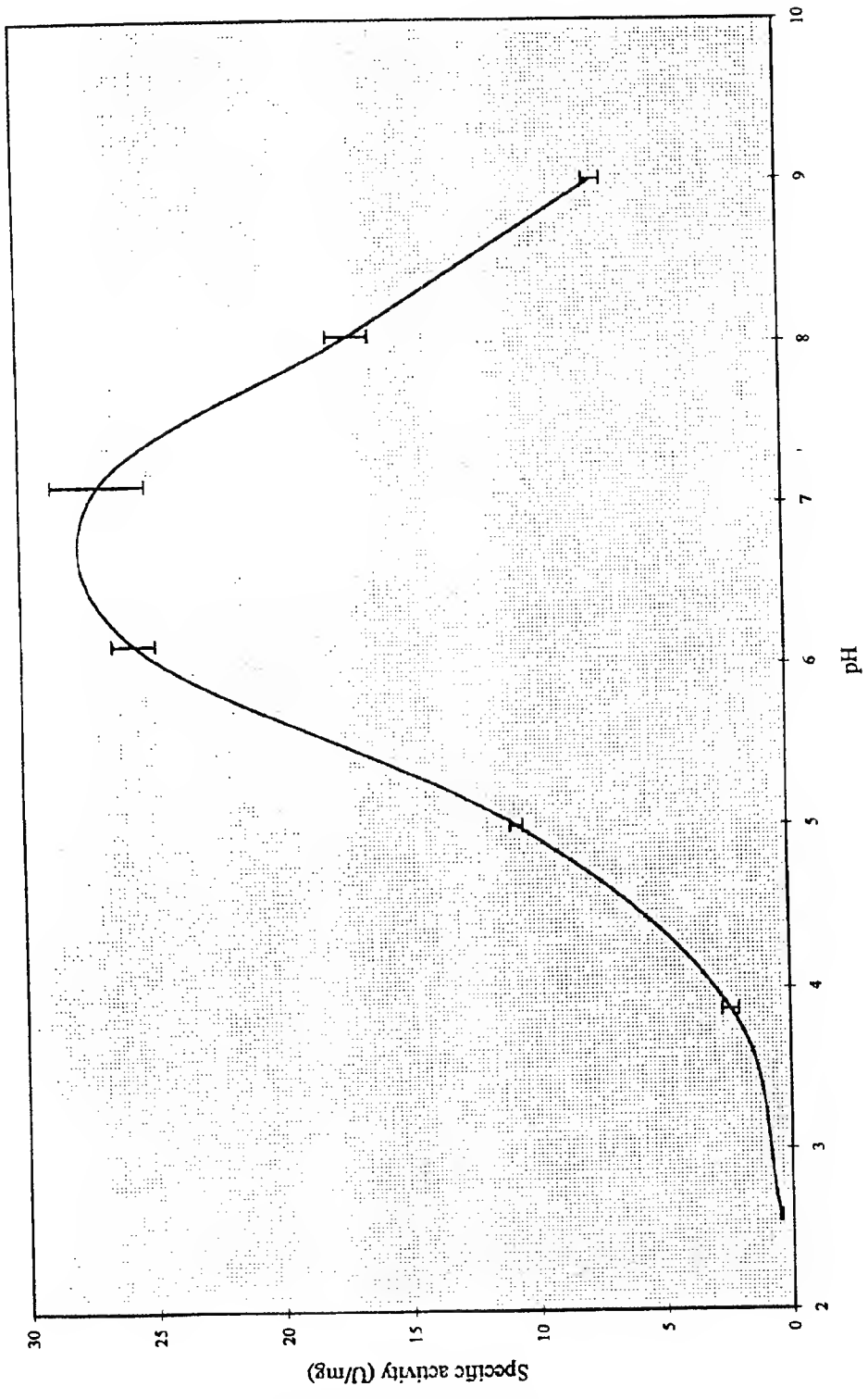

Figure 1: pH profile of the phenolic acid esterase using FAXX as substrate

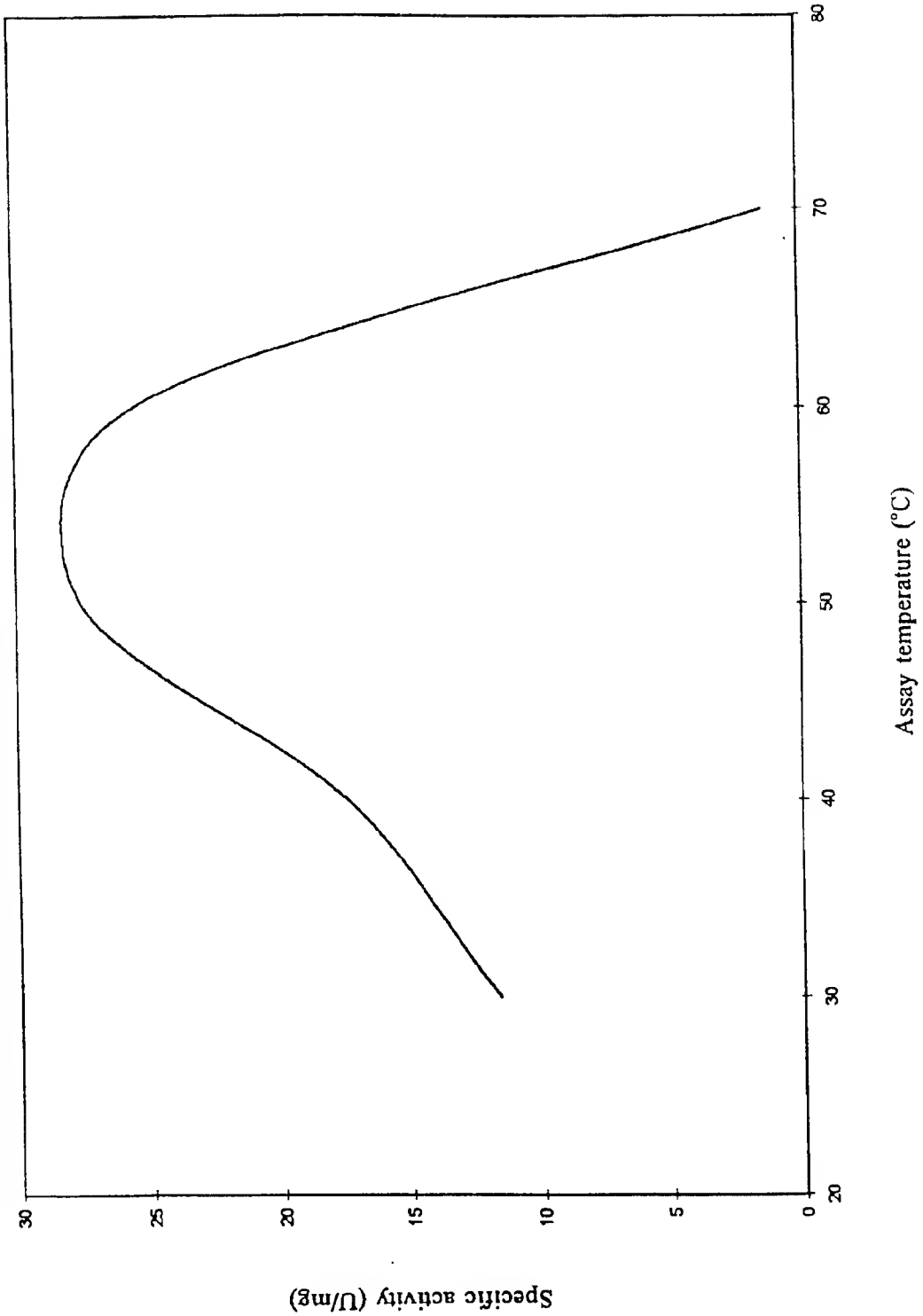

Figure 2: Temperature profile of the phenolic acid esterase using FAXX as substrate.